Chronic carbon monoxide inhalation during pregnancy augments uterine artery blood flow and uteroplacental vascular growth in mice.

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Running Title
Uteroplacental effects of carbon monoxide in mice

Summary Sentence
Chronic maternal inhalation of carbon monoxide in pregnancy doubled uterine blood flow and augmented uteroplacental vascular growth without altering maternal plasma sFLT1 or VEGF concentrations in mice.

Keywords: pregnancy, carbon monoxide, placenta, VEGF, sFLT1, preeclampsia

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Abstract

End-tidal breath carbon monoxide (CO) is abnormally low in women with preeclampsia (PE), while women smoking during pregnancy have shown an increase in CO levels and a 33% lower incidence of PE. This effect may be in part due to lowered sFLT1 plasma levels in smokers, and perhaps low-level CO inhalation can attenuate the development of PE in high risk women. Our previous work showed maternal chronic CO exposure (< 300ppm) throughout gestation had no maternal or fetal deleterious effects in mice. Our current study evaluated the uteroplacental vascular effects in CD-1 maternal mice who inhaled CO (250ppm) both chronically, gestation day (GD) 0.5 to 18.5, and acutely, 2.5hrs on each of GD 10.5 and 14.5. We demonstrated, using micro-ultrasound measurements of blood velocity and micro-computed tomography imaging of the uteroplacental vasculature, that chronic maternal exposure to CO doubled uterine artery blood flow and augmented uteroplacental vascular diameters and branching. This finding may be of benefit to women with PE, as they exhibit uteroplacental vascular compromise. The ratio of VEGF protein to its FLT1 receptor was increased in the placenta suggesting a shift to a more angiogenic state, however maternal circulating levels of VEGF, sFLT1, and their ratio were not significantly changed. Doppler blood velocities in the maternal uterine artery and fetal umbilical artery and vein were unaltered. This study provides in vivo evidence that chronic inhalation of 250ppm CO throughout gestation augments uterine blood flow and uteroplacental vascular growth, changes that may protect against the subsequent development of preeclampsia.
Introduction

Pre-eclampsia (PE) is a complication of pregnancy, characterized by the new onset of hypertension and proteinuria (43; 46). It is a life-threatening disorder, ameliorated only by the removal of the placenta, at which point maternal signs and symptoms usually resolve (43; 46). While the exact etiology of the disorder continues to be elusive, it is believed that PE begins with abnormal placental development, and progresses in some women, when coupled with maternal factors that affect endothelial function (43; 46). Although it is well-known that smoking in pregnancy can cause fetal complications, cigarette smoking continues to be a prominent external factor which reduces the risk of developing PE; several researchers, including us (58; 60) have reported similar findings (14; 16; 30; 57; 61). In fact, cigarette smoking in pregnancy reduces the incidence of PE by as much as 33% (16), and does so in a dose-dependent manner (22; 55; 61). Interestingly, pregnant women who use smokeless tobacco (i.e. Snuff) do not have the same reduction in PE as women who smoke cigarettes (55). The main difference is observed in the combustible elements of cigarette smoking; as nicotine and other components of these tobacco products are similar between them. Carbon monoxide (CO) is one such product of combustion found in cigarettes, increased in blood and end-tidal breath of those who smoke (52), but decreased in the end-tidal breath systems of women who develop PE (29).

We have previously shown in CD-1 mice (51), that maternal chronic exposure to CO (< 300ppm) throughout pregnancy does not negatively affect fetal growth or development. We measured fetal and placental weight, and compared resorption and implantation sites to control animals. In this study, maternal mouse blood-CO levels were comparable to those of women who smoke a pack of cigarettes per day or less, roughly 10% carboxyhemoglobin (%COHb) in the blood or lower. This study implicated the possibility of a dose of CO for further testing the hypothesis of its benefit in reduction in the incidence of PE.

The normal development of a placental vascular system is one of complexity and intricate communication with the maternal uterine vasculature. The trophoblast cells of the placenta invade and remodel the maternal spiral arterioles in the uterine wall, rendering them large conduits for blood, unresponsive to vasoactive factors (43). In women with PE, this process improperly takes place, affecting the uteroplacental vasculature. Studies utilizing Doppler waveforms in pregnancy have shown a
relationship between PE and an increased resistance index (RI) in the uterine artery (UtA) in both early and late gestations, possibly leading to the decrease in flow measured in the same vessel.

Similar findings were observed in the umbilical artery (UMB A) of fetuses in women with PE, with an increase in RI and pulsatility index (PI). Women who smoke in pregnancy display no difference in the RI of the UtA compared to non-smoking women, however, in this same group of women, some revealed a diastolic notch, indicative of resistance in the vessel. An increase in RI is also observed in the fetal UMB A of maternal smokers, a finding of negative nature for the developing fetus. A study conducted in pregnant sheep reported similar results when nicotine was administered to maternal sheep, indicating a possible causal role for nicotine in the effects of smoking on the uteroplacental Doppler measurements. In the current study, we chose to evaluate the effects CO on the uteroplacental vascular unit, to determine if similarities with nicotine were observed.

A pulsatile blood velocity is associated with hypoxic areas of the placenta, leading to the release of anti-angiogenic factors, one of which, soluble fms-like tyrosine kinase-1 (sFLT1) has come into light as a possible mediator in the development of PE. Measured in maternal serum, this molecule is normally elevated in pregnancy, but greatly increased (almost five-fold) in the serum of women with PE; a finding thought to play an important role in the development of PE. Its actions interfere with two very important angiogenic molecules of pregnancy, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). As a soluble form of the VEGF receptor, sFLT1 is capable of binding and inhibiting the actions of both VEGF and PlGF in pregnancy. Smoking in pregnancy leads to a decrease in plasma levels of maternal sFLT1 at mid gestation, therefore, we sought to determine if elevated CO levels could be responsible for this altered measurement, as this action could offer benefit to women with PE.

The objective of the present study was to determine the effect of 250 ppm CO on placental development, specifically evaluating changes in placental vascular parameters; protein markers affecting angiogenesis, VEGF and sFLT1, Doppler ultrasound measurements and gross vascular alterations.
Materials and Methods

All experimental procedures were approved by the Queen’s University Ethics committee (REB no. Smith 2007-052-Or) and carried out according to protocol of the Queen’s university animal care committee.

Animals and Husbandry

Female CD-1 mice (8-10 weeks old) were mated with males (6-8 weeks old) of the same strain overnight. The detection of a copulation plug was deemed gestation day (GD) 0.5. Mice were placed in a CO-dosing chamber and allowed food and water *ad libitum* until sacrifice, at GD 14.5. For a total comparison of n- values used for each of the study protocol sections, refer to Table 1.

Carbon Monoxide Exposure

CO was administered exogenously to maternal mice, either chronically throughout gestation, (beginning on GD0.5) or acutely (for 2.5 hours on each of GD 10.5 and GD14.5). Doses of 0 and 250ppm CO were administered in a sealed chamber as previously described (51). CO levels within the chamber were controlled at 250ppm, and verified using gas- solid chromatography. The air measured an average of 252.24 ± 14.19 (ppm ± SD) for chronic CO levels and following the half hour chamber equilibration time, 249 ± 13.42 for acute CO levels. We verified that 30 minutes was sufficient time to produce desired CO levels within the chamber (data not shown).

Three gestational days were used for procedural application; GD0.5 (baseline), GD10.5 (mature placental structure is established)(39) and GD14.5 (placental blood flow velocity is established)(10). Mice were kept within the chamber at all times that procedures were not taking place. Maternal weight was recorded throughout pregnancy and compared between groups as a measure of maternal health. The total number of live fetuses and resorptions per mouse were recorded.

Doppler Analysis

Mice were anesthetized with isoflurane gas (1-3%) and oxygen by face mask during ultrasound procedure (limited to an hour of anesthesia). Maternal heart rate was maintained between 480 and 530 beats per minute, by controlling the amount of isoflurane gas administered to the mouse. Maternal temperature was maintained between 36 and 38 degrees Celsius by a rectal temperature probe. The hair
on each mouse's abdomen was removed using a chemical hair remover (Nair) and ultrasound gel was warmed before use.

Control (n=12) and chronically (n=12) CO-exposed mice were imaged using Doppler technology on GD10.5 and GD14.5. Mice exposed to CO acutely (n=7), ultrasound analysis was performed before CO exposure and directly afterwards, on each of GD10.5 and GD14.5.

Using a Vevo 770 Ultrasound (Toronto, ON, Canada), we performed Doppler analysis of blood velocity for each maternal mouse (excluding those used for bloodwork) in the maternal UtA and four matched fetal UMB As and veins (UMB V) (two fetuses from each uterine horn). The maternal UtA blood velocity was measured below the bladder, as a direct branch from the internal iliac artery, as previously described by Mu. J et al. (39). The UMB A and UMB V measurements were taken directly exiting the placenta, as matched vessels. All blood velocity was measured at an angle less than 30 degrees between the Doppler beam and the vessel, using a RMV704 scanhead (Visualsonics, Toronto, Canada) operating at 20-60MHz. Data was collected and saved for analysis at a later date.

Analyses were performed in a blinded fashion using the instrument software package provided with the Vevo 770. For each waveform video, a minimum of 10 measurements were taken for the peak systolic (PSV) and end diastolic blood velocity (EDV). The maternal UtA and fetal UMB A measurements are presented as RI= (PSV- EDV)/ PSV. Fetal UMB V was compared using peak velocity measurements (cm/s).

**Necropsy and injection of contrast agent**

On GD 14.5, following ultrasound analysis, mice were anesthetized by intraperitoneal injection of 10mg/g of 2-2-2 Tribromoethanol (T48402, Sigma Aldrich, Canada). Contrast-infusion procedures were carried out as per Whitely et al. (54), with the following differences. We used 2-2-2 Tribromoethanol to anesthetize our animals. In addition, we used a different medium for creation of the contrast-infused placental specimens. As described by Rennie MY et al. (45), we infused a radio-opaque, silicone rubber X-ray contrast agent (Microfil; Flow Tech, Carver, MA) into the arterial system of the mouse, beginning in the thoracic aorta. The abdomen was opened to allow for visual tracking of the dyed rubber as it filled the arterial system, to ensure that it only filled as far as the labyrinth layer of the placentas; and not the venous system of the maternal mouse. It was left to polymerize for 1 hour following proper perfusion of
the vessels. During this time, total implantation sites, live fetuses and resorptions were recorded. The entire uterus was then excised and immersed in 4% paraformaldehyde (PFA) for 24hrs at 4 °C. At this point, uterine horns were either transferred to 1X phosphate buffered saline (PBS) for future setting, or immediately set in 1% agar prepared in 4% PFA. Specimens were mounted so as to elongate the uterine horn and expose the placenta and its vasculature to imaging.

**Micro-computed (micro-CT) tomography imaging**

All mounted samples were sent to the Hospital for Sick Children, Mouse Imaging Centre. As previously described (44; 45), three-dimensional images of each placenta were created and blinded for analysis, by the technologist in charge of the micro-CT. Vessel number and diameter, in addition to diameter length of the intervillous space were measured in a blinded fashion using the Amira Software package (Visage Imaging, San Diego, CA). A minimum of two placental images (one per uterine horn) were saved per mouse for analysis. Maternal UtA diameter was measured in each of the placental units imaged. Ten measurements were taken per image; therefore a total of 20 measurements were averaged per UtA in each mouse. For each placental unit imaged, a minimum of ten measurements of diameter were taken randomly throughout each vessel being analyzed. In each placental unit, the measurements were averaged for each vessel, and this value was compared in each of the respective CO treatment groups. The following arteries were analyzed: UtA, radial arteries, spiral arteries, and canals (See Figure 1 for a description of placental layers, and a comparison of control versus treatment groups). We defined radial artery branches and canal branches, as the point at which the vessel began to split into more than one vessel (Figure 1). The number and diameter of vessels at this point were also counted. A total of 6 measurements of the diameter of the intervillous space were taken and the mean of these were compared between placentas (Figure 1). For those maternal mice who were used in both the ultrasound analyses and the micro-CT analysis, blood flow in the UtA was calculated (control n=8, chronic CO n=5). Maternal mouse UtA mean velocity over a cardiac cycle (MV) was measured and used with the corresponding UtA diameter (d) measurement in the following calculation for blood flow = A•MV, or Blood flow= \( \pi \cdot \left( \frac{d}{2} \right)^2 \cdot MV \).
Blood Collection

Blood was collected from all mice on GD10.5 and GD14.5, but CO levels were only measured in a select group of mice for each experimental group (refer to table 1). Blood was collected from all conscious maternal mice on GD10.5 via the submandibular vein. Using a 5mm lancet (Golden rod, Braintree Scientific, MA), the vein was punctured and 5 drops of blood (roughly 100ul) were collected into microcentrifuge tubes containing 10ul of 1400U/ml sodium heparin (Sigma Aldrich, H0777) on ice. On GD14.5, upon reaching surgical plane of anesthesia, blood was collected by retro-orbital puncture, using a glass pipette, and transferred to a microcentrifuge tube containing 10ul of 1400U/ml sodium heparin (Sigma Aldrich, H0777), on ice.

CO Measurement

CO was measured in a separate set of mice from those imaged using Doppler ultrasound (Table 1). Hemoglobin levels were measured using a Hemocue, Hb 201 (Hemocue, Sweden). Maternal blood was measured for CO level as previously described(51) using gas –solid chromatography (Peak Performer 1, Palo Alto, San Francisco, USA), and blood CO levels were expressed as %COHb.

Plasma Separation for ELISA assay

All blood was centrifuged for 15minutes at 16 000 rpm, and plasma was removed and stored at -80 degrees Celcius until future analysis using.

Placental Protein measurements

A random sample of mice (control n=5, chronic CO n=5, acute CO n=6) were not perfused, to allow for placental protein analysis. Placentas were removed from the uterine tissue, immediately frozen in liquid nitrogen and maintained at -80° Celcius. Total placenta numbers used for protein analysis is shown in Table 1.

Total Protein Analysis

Each placenta was homogenized in 1%Triton X, 1XPBS buffer using a dounce homogenizer. The homogenate was centrifuged at 14000rpm for 20min at 4°C. The supernatant was removed to a fresh
microcentrifuge tube and maintained on ice. A 1 in 2 dilution of the sample in the same buffer was prepared and protein levels were measured using a standard CD Lowry Assay (Bio Rad, Sigma). Further dilutions were prepared to ensure each sample was 2ug protein/ul of sample. All sample loading was verified using western analysis (data not shown).

**Enzyme-linked immunosorbent assay (ELISA) analysis**

Measurement of protein level sFLT1 (Quantikine MVR100, R &D systems, Canada), and free VEGF (Calbiochem, Q1A52) was completed using a sandwich ELISA in duplicates, for both maternal plasma and the placental homogenates. The sFLT1 ELISA is not specific to the soluble VEGF receptor, and also measures the transmembrane VEGF receptor (VEGFR1). In the plasma, the only measured VEGFR should be the soluble form, therefore we refer to this protein as sFLT1. In the placenta, both soluble and membrane-bound VEGFR1 may be present, therefore we refer to this protein measurement as total VEGFR1. Each ELISA was standardized to its respective control protein supplied with the kit materials. Maternal plasma was diluted 1/5 for the VEGF ELISA and 1/10 for the sFLT1 ELISA, using the respective diluent provided. Placental homogenates were used at 2ug/ul for the VEGF ELISA (total 100ug of protein loaded per sample in duplicate) and further diluted 1/5 for the sFLT1 ELISA (total of 20ug of protein loaded per sample in duplicate).

**Statistics**

Statistical analysis of placental vessel diameters and blood flow comparison was completed using a Student’s t-test; significance identified at p<0.05. All remaining statistics were completed using a one way analysis of variance and post-hoc Tukey test (Graphpad Prism Version 6.0). A p value <0.05 was deemed significant and all results are reported with standard deviation (SD). All n values used in the analysis are shown in Table 1.
RESULTS

Maternal CO exposure during pregnancy did not affect fetal resorption number or maternal / fetal weight in comparison to control

In each of the experimental groups, maternal mice appeared to be healthy throughout gestation, with no difference in the change (p>0.05) in maternal weights (g ± SD) from GD0.5 to GD14.5 between the groups (control 16.4 ± 1.6, chronic CO 15.4 ± 1.6, acute CO 15.4 ± 1.3). Assessment of litter size was determined for each mouse, evaluating the live fetus number, in addition, this number as a fraction of the total implantation sites, and no difference was observed between mice exposed to CO and control (Table 2). Further, for each maternal mouse, fetal resorptions were calculated as a fraction of total implantation sites, and again no differences were observed between the groups (Table 2). As an indication of CO exposure, levels of CO were calculated as a percentage of total hemoglobin and presented as %COHb. For mice exposed to chronic CO, %COHb levels were significantly higher than control (Table 2). Levels of CO in mice exposed to CO acutely were calculated on GD14.5 only, and again were significantly higher than control mice (Table 2). Chronic and acute CO exposed mice did not differ in the level of their %COHb levels. Levels up to 14%COHb have been measured in women who smoke while pregnant(32), and while a direct comparison cannot be made between humans and mice, our study maintains a level of COHb within a close range of pregnant smokers.

Increased vessel diameters of arteries in uteroplacental units of contrast- infused specimens

Micro-CT images of the placental units allowed for diameter measurements of the vascular tree from the maternal UtA to the placental intervillous space (Figure 1). Maternal chronic CO exposure led to an increased vessel diameter in the maternal uterine artery, in addition to the spiral arteries and the branching vessels of the radial arteries and the canals (p<0.05) (Figure 2). The intervillous space diameter (mm) was not statistically different (p>0.05) between CO- exposed and control groups, 5.54 ± 0.36 vs. 5.69 ± 0.61 respectively. In addition to vessel diameter, a quantification of vessel number was assessed for radial arteries, canals and each of their branching vessels, Between control and chronic CO-exposed mouse groups, the number (±SD) of radial artery vessels, 1.75 ± 0.71 and 1.75 ± 0.45 respectively and canals, 3.63 ± 1.3 and 3.08 ± 1.08 respectively, were not significantly different (p>0.05). The number of radial artery branches was significantly increased in the chronic CO exposed group (11.08
± 4.55) versus the control group (7.25 ± 2.44) (p<0.001), as was the number of canal branches, chronic (CO 7.55 ± 0.45) versus control (2.25± 2.71) (p<0.001).

**Uteroplacental blood flow velocity was not different between CO-exposed mice and control, but maternal blood flow was significantly higher in the group exposed to chronic CO**

Blood flow velocity was measured in each of the maternal uterine arteries, in addition to the fetal umbilical arteries and veins. Data are presented for each of these vessels on both GD10.5 and GD14.5, and peak systolic velocity (PSV) is displayed in all cases, in addition to RI value for arterial vessels (Table 3). Doppler analysis demonstrated no significant change in the PSV or the RI of the maternal UtA or the fetal UMB A of maternal mice exposed to chronic or acute CO compared with control (p>0.05). This finding was true on both GD10.5 and GD14.5, in addition to the acute exposure before and after CO (p>0.05). UMB V peak velocity (cm/s) did not differ between experiments. It is important to note that in order to complete Doppler measurements, mice are anesthetized with isoflurane by nose inhalation. In doing so, the level of maternal CO decreases more quickly than in general room air. Our unpublished data suggests that in normal room air, the half- life for maternal mouse %COHb is 88minutes and for a mouse on isoflurane, the half- life is reduced to16.5minutes. Special care was taken to ensure that measurements were taken within thirty minutes, to decrease the effect of isoflurane on maternal %COHb.

Although maternal blood flow velocity was not different between groups, blood flow calculations led to different results. Calculations for blood flow were possible due to the vessel diameter measurements using the contrast- infused specimens for chronic CO-exposed mice and control. Maternal mouse UtA blood flow was significantly increased in those mice exposed to CO (Figure 3).

**Placental and maternal plasma protein ratios of sFLT-1 to VEGF are shifted towards an angiogenic balance in CO-exposed animals versus control**

Maternal plasma measurements for both protein VEGF and protein sFLT1 levels are shown using a box and whisker plot in Figure 4a and b respectively. No difference (p>0.05) was noted between control and chronic or acute CO- exposed mice in VEGF levels (GD10.5 or GD14.5), though a small increasing trend was noted in the CO exposed mice compared to control. Plasma sFLT1 levels were also not different between the three groups, (GD10.5 or GD14.5), though a trend towards a decrease in
protein levels was observed in acute versus control mice. The ratio of sFLT1 to VEGF was not different
between groups on GD10.5, however, on GD14.5, though no change was observed between control vs.
chronic CO-exposed mice (p>0.05), a significant decrease (p=0.008) between control and acutely
exposed mice was observed.

Placental protein levels (Figure 5) followed a similar pattern to maternal plasma for each of the
respective protein levels. A trend (p=0.092) towards increasing placental VEGF levels was observed
from maternal dams exposed to chronic CO; an increase (p=0.003) was observed in the acutely exposed
mice. Though no significance was measured in the total VEGFR1 protein levels, a decreasing trend in
protein levels was determined in those mice exposed to acute CO. Finally, the ratio of total VEGFR1:
VEGF was significantly lower in both the chronically-exposed (p=0.008) and acutely CO-exposed
(p=0.0008) maternal mice compared to control.
Discussion

PE is a leading cause of maternal morbidity and mortality worldwide\(^\text{43}\) and it continues to claim the lives of upwards of 50,000 women a year in developing countries\(^\text{18}\). There is no cure for the disorder itself, until delivery occurs\(^\text{43}\). It has been shown that women with PE have significantly lower end-tidal breath CO levels compared to those with healthy pregnancies\(^\text{29}\). Therefore, it is possible that elevated CO levels in women who smoke during pregnancy may contribute to the reduced risk of developing PE. While this study aimed to evaluate the effects of CO exposure in pregnancy, ultimately as a possible mechanism for the observed reduced incidence of PE in women who smoke, the specific goal of this study was to determine if CO altered the vascular system of the uteroplacental unit.

We used healthy, pregnant CD-1 mice, without induction of PE-like symptoms, to allow for a clear observation of placental alterations due to maternal CO exposure. The CO dose of 250ppm was chosen based on our previous work in CD-1 mice\(^\text{51}\) which demonstrated that below 330ppm CO, maternal exposure does not lead to fetal alterations of growth and development. Further, exposure to 250ppm CO led to maternal mouse CO levels of 12.65\%COHb, similar to maternal \%COHb levels in women who smoke roughly a pack of cigarettes per day during pregnancy; measured in our lab as high as 9.85\%COHb (unpublished) and by others as 14\%COHb\(^\text{31}\). As well, other research groups have evaluated 250ppm CO in animal models studying its anti-inflammatory effect \(^\text{41; 48}\) and tissue graft transplants \(^\text{2; 12; 13}\); for a more complete list of experiments, refer to the review by Motterlini and Otterbain \(^\text{38}\).

We evaluated placental vascular changes on GD 10.5 and GD 14.5 in order to test the effect of CO on the placental vascular development (GD10.5- implantation is complete, vascular invasion commences and the blood begins to cross the placenta to the fetus\(^\text{1; 47}\), GD 14.5- placental vascular flow velocity is established and the first quiescent phase of fetal vasculature occurs\(^\text{15}\)). We demonstrated that maternal chronic exposure to 250ppm CO throughout pregnancy increases placental vascular branching and vascular diameter. The diameters of almost all uteroplacental vessels were enlarged significantly, while branching was augmented in the radial arteries and the canals, directly above the placental labyrinth. The increase in branching is especially curious, as these vessels (canals and radial arteries) are quite different in vascular character. Radial arteries are very much vascular in nature,
while the placental canals are more so channels for blood travel, with no endothelium or smooth muscle. The molecular mechanism of CO on each of these vessels remains to be elucidated. The altered placental growth pattern offers some adaptation control due to an environmental exposure to CO. The micro-CT imaging results suggests that CO-induced remodelling could lead to increased oxygen and nutrient delivery from maternal to fetal vessels. In a pathological pregnancy complication such as PE, the placental nutrient delivery is compromised; increased branching and vessel size could reduce some of the negative effects.

CO has been shown to decrease placental apoptosis and to elevate placental VEGF protein and mRNA(19). We measured protein levels of total VEGFR1 and VEGF and our data values were similar to those reported in the literature for pregnant mice, for both VEGF(56) and total VEGFR1(33; 56). We confirmed a significant increase in protein VEGF levels in placentas from maternal dams exposed to acute CO, while an increase (not significant) was also observed in those placentas of maternal chronic CO exposure. Perhaps the CO effect is more strongly related to a decreased sFLT1 release, thus increasing the VEGF bioavailability. Indeed, it has been previously shown that HO-1/CO pathway is capable of decreasing the sFLT1 release from human umbilical vein endothelial cells in vitro (17) and from rat placental explants in both hypoxic and normoxic conditions(21). We demonstrated that the ratio of placental total VEGFR1 to VEGF was significantly reduced in both chronic and acutely-exposed animals, indicating a possible mechanism for the placental vessel changes noted. The placentas of women who smoke in pregnancy have been reported as larger in size, with an increase in branching of the vascular tree (42); the authors suggest that this alteration is an adaptation response, increasing surface area for nutrient transfer. This adaptive angiogenesis was similar to our findings. It is clear that CO plays a role in angiogenesis, a process necessary for placentation and may account for the CO-induced increases of placental vessel branching in the placentas of women who smoke in pregnancy. Several researchers have reported on the importance of CO and its parent enzyme heme oxygenase, (HO) in pregnancy (17; 36; 40; 50). In a recent study(59), it was shown that without HO-1, placental implantation is compromised, placenta structure is altered and fetal loss is increased significantly. Interestingly, in HO-1 null mice, maternal CO exposure (50ppm) diminished fetal loss and restored the placental trophoblast cell viability; placentas were structurally healthy. In PE, the placenta is
compromised at the maternal-fetal interface, where spiral artery remodelling is impaired (46). It is known that HO-1 is expressed at the maternal-fetal interface and its production of CO through heme catabolism may be important for implantation, placental development and fetal survival (as shown in a murine model) (59). Perhaps a deficiency in this pathway is a part of the progression of some women to develop PE. Our study provides a possible mechanism whereby the exposure to CO can alter the placental development. Translated to an animal model of PE, CO could potentially ameliorate or attenuate the clinical symptoms of PE.

No significant difference between control and CO-exposed groups was observed in either of the protein levels measured for VEGF and sFLT1 in maternal plasma. Similar results were observed when the ratio sFLT1 to VEGF was calculated for chronic CO-exposed maternal mice; however the ratio was significantly lower for those exposed to acute CO. This could be due to a decreased sensitivity in the mice exposed to chronic CO exposure. In a study conducted by George et al. maternal rat sFLT1 levels were increased in rats by implanted mini osmotic pumps, with a group of rats also receiving an inducer of the enzyme producing CO. VEGF levels were only increased in rats who were first infused with sFLT levels, followed by induction of the CO system. No alterations were observed when control mice were exposed to increased CO levels. Therefore, it is possible that in our study, exposure to CO would not affect maternal plasma sFLT1 or VEGF levels until alterations in normal levels were first induced. One of the goals of using CO as a possible future therapeutic in patients with PE would be to lower plasma sFLT1 levels. In this regard, future studies would be necessary to investigate the effect of repeat exposure to short term CO levels on maternal plasma sFLT1.

The Doppler blood velocity findings were surprising, as we expected CO, a known vasodilator, to increase the blood velocity in all vessels measured. It could be, that in control mice, vessels are already maximally dilated and little change would be observed. Perhaps the exposure to CO in pregnancies of complication, such as PE, would offer different results. Though no change was observed, this finding was able to highlight that CO is not responsible for the increased RI and PI in the UMB A observed in the fetal vasculature of maternal smokers. This is of great importance, as an increased RI negatively impacts the nutrient transfer to the fetus.

Though the maternal UtA blood velocity was not different in maternal mice exposed to CO versus
control, the blood flow was significantly increased. This makes sense, due to the increased UtA diameter measured in the contrast-infused placentas. The equation for blood flow demonstrates that an increased vessel size with no change in mean blood velocity, would lead to an overall increase in flow, or a larger amount of blood travelling per minute. By using CO to augment uterine blood flow rates, it may be possible to reverse or prevent this risk factor, decreasing the development of PE.

In addition to our previous work(51), others have shown that in vivo exposure to low-dose CO or induction of HO-1, is beneficial to both mother and fetus(es). El-Mousleh et al.(19) have shown that low dose (50ppm) exposure to CO in pregnancy in early gestation rescues intrauterine growth restriction in mice, in addition to decreasing placental apoptosis, reduces pro-inflammatory cytokines, and exhibits pro-angiogenesis in the placenta. Further, the same research group has demonstrated that IUGR seen in HO-1 null mice can be rescued by maternal exposure to CO(59). The exact mechanism by which this occurs has not been determined. However, it is possible that maternal exposure to CO would reduce the oxidative stress experienced by the placenta, potentially decreasing the free radical production and oxidative stress-induced apoptosis as observed in different organ systems(62) and in placental ischemia-reperfusion models(5; 21). Our study did not evaluate the effect of CO on placental oxidative stress, and future work should aim to report on these findings. In addition, George et al.(20) have shown that HO-1 induction attenuated ischemia-induced hypertension in rats, and shifted the placental sFLT1/VEGF ratio to one of increased angiogenesis, previously observed in a rat reduced uterine perfusion pressure placental ischemia model.

There are other potential beneficial effects of both HO-1 and CO in placental development and fetal survival. Several studies have evaluated the effects of HO-1 in pregnancy, and it is clear that this enzyme is necessary for proper implantation and placental development. It would make sense then, that increasing one’s CO levels could rescue some of the deleterious effects associated with PE. Perhaps women who smoke cigarettes during pregnancy are able to replace the decreased levels of CO present in women with PE(8). It is still unclear how, if true, low doses of CO may aid in the decreased incidence of PE, however there are several possible mechanisms for this to take place. In fact, endogenous and exogenous CO has been shown to decrease anti-angiogenic factor release(17), support angiogenesis(53), vasodilate blood vessels(6) and suppress inflammation(19). In placental tissue
specifically, CO, at physiologic levels, has shown decreased inflammation and apoptosis(5), in addition to increased vasodilatory effects(6). Each of these properties could be helpful in reducing the development of PE.

Unfortunately, smoking does not completely ameliorate the development of PE. Cigarette smoking appears to have less of an effect on the development of PE in more severe forms of the disorder(55) and women who smoke and develop PE are at an increased risk for adverse pregnancy outcomes(37). As Karumanchi and Levine(27) have postulated, perhaps there is a threshold of angiogenic balance, which when crossed, leads to the development of PE. Further, it is possible that CO in cigarette smoke reduces the incidence of PE by altering the angiogenic/ anti-angiogenic balance. However, in more severe forms of PE, it would take more of a reduction in the anti-angiogenic molecules to prevent the development of PE. Karumanchi and Levine(27) have further proposed that smoking in pregnancy more frequently reduces the incidence of mild forms of PE, thus increasing the proportion of women who develop more severe forms of PE in women who smoke.

**Perspectives and Significance**

Our study provides evidence for the effects of CO in pregnancy, and the role it might have in attenuating the signs of PE. We are the first to use placental contrast-infused specimens to evaluate the vascular effects of maternal mouse exogenous CO exposure throughout pregnancy. With a focus on the placental development, we showed that maternal inhalation of 250ppm CO increased angiogenesis in the placenta, in addition to gross vessel size, possibly as a result of the concomitant increase in VEGF compared with VEGFR1. These findings are important, as they provide a possible explanation for the lower incidence of PE among smokers; given that these patients' CO levels are much higher than non-smokers. More importantly, this study supports the possibility of use of CO as a therapeutic in the treatment of PE.
Acknowledgements

We wish to thank Jalna Meens, Queen’s University, for aiding us with some of the ultrasound Doppler measurements and for guidance in teaching C.C.V proper techniques in Doppler data acquisition. Further, we wish to thank Lisa Xiu, from the University of Toronto, for performing all micro CT imaging of the contrast-infused placental specimens.

Funding

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Figure 1: An iso-intensity surface rendering from micro-computed tomography of a CD-1 mouse placenta, GD 14.5, from both a control and maternal CO-exposed mouse. A) Representative image of a control placenta, with areas of the placenta identified, as defined in our protocol. B) Representative image of placenta from a chronic CO- exposed maternal mouse. Increased branching of the radial arteries can be seen (white arrow) in comparison to A.

Figure 2: Measurements of placental vessel diameters in those from maternal dams in control groups and those exposed to chronic CO. Imaging of the contrast- infused placental specimens was completed by micro- computed tomography and vessel measurements are displayed. Maternal exposure to chronic CO increased the vessel diameter in the uterine artery, the spiral arteries and the branching vessels of the canals and the radial arteries, of the uteroplacental unit compared to control (*p<0.05, **p<0.01, ***p<0.001).

Figure 3: Maternal uterine artery mean blood flow between chronically CO- exposed maternal mice and control mice. Using the uteroplacental vessel measurements for uterine artery diameter and the Doppler mean velocity over a cardiac cycle, the mean blood flow was determined. Although Doppler blood flow was not different between the two groups, the maternal uterine artery diameter was increased in those mice exposed to CO, thereby increasing the blood flow in the vessel (* p<0.05).

Figure 4: Maternal plasma protein levels: vascular endothelial growth factor (VEGF) and its soluble receptor. Maternal plasma was collected and compared on GD10.5 and GD14.5 for mice in control, chronic CO-exposed and acute CO-exposed groups. A) Comparison of maternal plasma VEGF protein levels, not significant (p>0.05). B) Comparison of soluble fms-like tyrosine kinase -1 (the soluble VEGF receptor, sFLT1) protein in maternal plasma; no significance between groups (p>0.05). C) Comparison of the ratio of sFLT1 to VEGF in maternal plasma; significance observed in the acute CO-exposed animals only (p<0.05).

Figure 5: Placental (GD14.5) protein levels for VEGF and its soluble anti-angiogenic receptor. A) VEGF protein levels were significantly (p<0.05) different in acutely- exposed animals versus control, but not in chronic CO- exposed animals. B) Levels of total VEGFR1 were not different in any of the groups of mice (p>0.05). C) Both chronic and acute CO- exposed animals resulted in a decrease in anti angiogenic total VEGFR1 to angiogenic (VEGF) protein (p<0.05).
Table 1. Explanation of n-values for each section of the study and used for statistical analysis.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Control</th>
<th>Chronic CO Exposure</th>
<th>Acute CO Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Mice Used</td>
<td>17</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Maternal blood CO measurement (GD10.5)</td>
<td>5</td>
<td>5</td>
<td>n/a</td>
</tr>
<tr>
<td>Maternal blood CO Measurement (GD14.5)</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Doppler Analysis (maternal mice)</td>
<td>12</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Doppler Analysis (fetus number)</td>
<td>48</td>
<td>48</td>
<td>28</td>
</tr>
<tr>
<td>Micro-Computed Tomography (maternal mouse number)</td>
<td>5</td>
<td>6</td>
<td>n/a</td>
</tr>
<tr>
<td>Micro-Computed Tomography (total placental units imaged)</td>
<td>18</td>
<td>12</td>
<td>n/a</td>
</tr>
<tr>
<td>Protein analysis (maternal plasma samples)</td>
<td>12</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Protein analysis (placenta samples)</td>
<td>11</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2. Maternal plasma CO levels and fetal litter numbers.

<table>
<thead>
<tr>
<th></th>
<th>Maternal Plasma %COHb (± SD)</th>
<th>Healthy Fetuses</th>
<th>Resorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GD10.5 (n=5)</td>
<td>GD14.5 (n=5)</td>
<td>Mean of (Live fetuses/ total implantation sites per mouse) (± SD)</td>
</tr>
<tr>
<td>Control</td>
<td>0.53 ± 0.13</td>
<td>0.82 ± 0.23</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>Chronic CO</td>
<td>11.34 ± 5.58**</td>
<td>13.24 ± 2.98**</td>
<td>0.92 ± 0.14</td>
</tr>
<tr>
<td>Acute CO</td>
<td>n/a</td>
<td>11.39 ± 4.02**</td>
<td>0.98 ± 0.07</td>
</tr>
</tbody>
</table>

All values are compared to control and presented as (±SD) (*p < 0.05 and **p<0.001).
Table 3: Doppler blood velocity measurements in the maternal uterine artery and fetal umbilical artery and vein.

<table>
<thead>
<tr>
<th></th>
<th>UTERINE ARTERY</th>
<th>UMBILICAL ARTERY</th>
<th>UMBILICAL VEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GD10.5</td>
<td>GD14.5</td>
<td></td>
</tr>
<tr>
<td>PSV (cm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.57 ± (3.71)</td>
<td>22.05 ± (9.59)</td>
<td></td>
</tr>
<tr>
<td>Chronic CO Exposure</td>
<td>17.57 ± (9.50)</td>
<td>24.19 ± (7.70)</td>
<td></td>
</tr>
<tr>
<td>Before Acute Exposure to CO</td>
<td>19.57 ± (5.4)</td>
<td>23.91 ± (6.21)</td>
<td></td>
</tr>
<tr>
<td>After Acute Exposure to CO</td>
<td>20.29 ± (4.1)</td>
<td>23.49 ± (5.84)</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>0.64 ± (0.12)</td>
<td>0.54 ± (0.04)</td>
<td></td>
</tr>
<tr>
<td>PSV (cm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.65 ± (0.63)</td>
<td>7.07 ± (1.98)</td>
<td></td>
</tr>
<tr>
<td>Chronic CO Exposure</td>
<td>3.20 ± (0.42)</td>
<td>8.99 ± (2.00)</td>
<td></td>
</tr>
<tr>
<td>Before Acute Exposure to CO</td>
<td>3.32 ± (0.13)</td>
<td>8.76 ± (0.22)</td>
<td></td>
</tr>
<tr>
<td>After Acute Exposure to CO</td>
<td>3.34 ± (0.19)</td>
<td>9.19 ± (1.91)</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>0.82 ± (0.19)</td>
<td>0.91 ± (0.40)</td>
<td></td>
</tr>
<tr>
<td>Peak velocity (cm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.70 ± (0.27)</td>
<td>3.69 ± (0.78)</td>
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</tr>
<tr>
<td>Chronic CO Exposure</td>
<td>1.74 ± (0.47)</td>
<td>3.39 ± (0.69)</td>
<td></td>
</tr>
<tr>
<td>Before Acute Exposure to CO</td>
<td>1.56 ± (0.26)</td>
<td>4.32 ± (1.69)</td>
<td></td>
</tr>
<tr>
<td>After Acute Exposure to CO</td>
<td>1.70 ± (0.83)</td>
<td>4.62 ± (1.00)</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

All data is presented (±SD). Statistical significance was set at *p<0.05. Control maternal mice n=12, Chronic CO maternal mice n=12, Acute CO maternal mice n=7. For each maternal mouse imaged, 4 of their respective fetuses were imaged for umbilical artery and vein measurements. PSV= peak systolic velocity and Resistance Index (RI) = (PSV- EDV)/PSV.
Uterine Artery

Radial Arteries

Radial Artery Branching

Spiral Arteries

Canal

Canal Branching

Intervillous Space
Maternal Uterine Artery Blood Flow (ml/min)

Control

Chronic CO

*